Research Proposal:

ADHERENCE OF RAG-1 TO HYDROCARBON AND HYDROPHOBIC SURFACES DURING GROWTH

Submitted To:

Dept. of Microbiology, Tel-Aviv University

Mel Rosenberg

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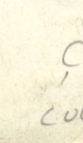
LPS markers:
KDO
heptose
Hydroxy FA
- specific monosaccharide
- amino sugars

TNS = toluidinyl naphalene 6 sulfonate
surfactive - effective - reduces ~~surface~~ interface tension
efficient - small conc - needed.

glyoxylate



KDO - 3 deoxy - D-manno-octulosonic acid

INTRODUCTION

The adherence of bacteria to a wide variety of inorganic surfaces has been well established, both in natural environments and in the laboratory (1 - 4). The advantage gained by "sticky" bacteria is perhaps most evident under circumstances in which the cells are able to adhere to surfaces which can provide necessary nutritional sources for metabolism and growth.

One interesting and important example of this phenomenon is the growth of hydrocarbon utilizing microorganisms on the surfaces of insoluble liquid and solid hydrocarbon (5). Growth of bacteria and yeast on hydrocarbon as a source of carbon and energy has received increasing attention in recent years. Potential applications are sought in such varied fields as food technology (6) and ecology (7). In addition to providing an inexpensive source of single cell protein, hydrocarbon degrading microorganisms and their extracellular products may provide a biological solution to oil pollution problems.

The adherence of bacteria to hydrocarbon may be viewed as one example of the more general phenomenon of interactions between bacteria and hydrophobic, non-wettable, hydrophobic surfaces. Other examples include the adherence of sulfur-oxidizing bacteria to insoluble sulfur particles (8,9) and the attachment of marine bacteria to a variety of hydrophobic surfaces (10-12).

Studies on the growth of microorganisms through direct contact with the bulk hydrocarbon phase are generally complicated by emulsion formation during growth (7). Many hydrocarbon degrading yeast and bacteria release emulsifying agents which "pseudo-solubilize" the hydrocarbon phase in the form of microscopic droplets while the culture is being mixed. Reports on the effects of endogenous production or exogenous addition of emulsifying agents on the degree of direct contact between the cells and the bulk hydrocarbon phase are often ambiguous and sometimes contradictory (13 - 18). A better understanding of the relationship between emulsifier synthesis and direct contact between the cells and the hydrocarbon phase is thus necessary.

The more general problem of how wettable bacteria are able to attach and adhere to non-wettable surfaces is to-date somewhat enigmatic. A better understanding of this general phenomenon may contribute to our ability to combat various problems, such as those related to marine biofouling (10).

In the proposed research, the characteristics and mechanism of adherence of the marine bacteria, RAG-1 to insoluble alkanes during growth will be studied. RAG-1 grows on higher alkanes as well as various non-hydrocarbon sources of carbon and energy (19). During growth on ethanol, acetate and hexadecane, RAG-1 synthesizes and releases a viscous, acidic polysaccharide of high molecular

Ilana's Method:

1) grow in C_{24} coated vials w/ P^{32}
2) Filter thru Whatman 1, add filter back to vial
FILTRATE
3) 5% TCA + cold P_i + PP_i filtered thru Millipore
+ washed 4 more times
4) rinsed w diethyl ether, counted
FLUID (sup)
5) 10% TCA + cold P
washed once w diethyl ether.

weight (about 10^6) which has emulsifying properties (20 - 22). This macromolecule, termed emulsan, differs from other emulsifying agents which have been isolated from hydrocarbon degrading micro-organisms in its large size, its structure, and the fact that it is a relatively poor surfactant. The physical and chemical properties of emulsan are currently under investigation in this laboratory.

MAJOR GOALS

It is intended that the proposed research will:

1) contribute to our understanding of adherence phenomena involving bacteria and hydrophobic surfaces;

2) examine the role of the outer cell surface in the adherence of RAG-1 to the bulk hydrocarbon phase;

3) elucidate the dependence of bacterial adherence on metabolism in the system to be studied;

4) generate a model which will account for the attachment and growth of bacteria on the bulk hydrocarbon phase.

PRELIMINARY WORKING MODEL: FACTS AND HYPOTHESES

1) The marine bacteria UP-2, has been observed to adhere to and grow on both liquid (hexadecane) and solid (tetracosane) n-alkanes through direct contact with the bulk hydrocarbon phase (5, 19). RAG-1 has also been observed to adhere to solid and liquid n-alkanes during growth on these substrates.

2) During growth of RAG-1 on hydrocarbon in the absence of mechanical agitation, the great majority of the cells will grow through direct contact with the bulk hydrocarbon phase.

3) Antibodies raised against emulsan cross-react with wild-type RAG-1 cells. This indicates that emulsan is present on the exposed cell surface prior to its release.

4) Emulsan contains fatty acid residues which are covalently bound to the sugar backbone (23). The fatty-acid composition of emulsan has been shown to be dependent upon the carbon and energy substrate on which the cells are grown.

It is postulated that cell-bound emulsan plays a decisive role in the process by which RAG-1 is able to adhere to the hydrocarbon surface. It is further suggested that emulsan-bound fatty-acid residues may be exposed at the outer cell surface,

thereby increasing the hydrophobicity of the cell exterior, and enhancing the cell's ability to bind to the hydrophobic surface. Fatty-acid composition of the cell surface has been shown to have an effect on the ability of yeast cells to bind emulsified hydrocarbon (24, 25).

look up Fiechter

Fiechter

5) It is possible to show that the adherence of RAG-1 to the hydrocarbon surface is a time-dependent process, and that its attainment requires cellular metabolism. It should also be possible to show that once the cells adhere, direct contact need not be maintained by the expenditure of cellular energy.

exopolysacc synthesis stimulated by excess carbohydrate + limiting PNS

6) Emulsan synthesis has been shown to be dependent to a certain extent on growth conditions. It is thus assumed that emulsan synthesis is subject to cellular regulation and can be inhibited under certain conditions.

7) Microscopic observation of adherent cells of both RAG-1 and UP-2 during growth on solid hydrocarbon reveals that the cells are not evenly distributed over the exposed hydrocarbon surface, but are concentrated in densely colonized patches, leaving other surfaces relatively bare.

It is postulated that the dense patches are microcolonies, i.e., the progeny of single cells which grew and divided without ever leaving the hydrocarbon-water interface.

PREDICTIONS AND EXPERIMENTAL APPROACH

1a. Conditions which repress or inhibit emulsan synthesis should also lower the degree of adherence.

Experimental - An accurate quantitative assay for cells bound to solid, bulk hydrocarbon has recently been developed (5). In addition, efforts will be made to develop a simpler method for enumerating bound cells. In the envisioned system, the hydrocarbon substrate will be evenly adsorbed to a membrane filter, which will be suspended in liquid medium. At any given time, one or a number of such filters can be removed from the aqueous medium, washed, clarified, and the bacteria bound to the filters counted either visually or using existing fluorescent staining techniques (26, 27). The number of cells in the aqueous phase can be determined by viable counts.

The concentration of emulsan in the medium can be quantitatively determined by an existing assay (21).

The effect of growth conditions on adherence and emulsan synthesis will be studied for parameters known to have an effect on polysaccharide synthesis by bacteria (28 - 32): nitrogen, phosphate, and sulfate concentration, and growth temperature.

1b. Adherence should be inhibited by hydrolysis of exposed fatty acid esters.

Experimental - Cells will be exposed to brief alkaline hydrolysis as well as treatment with esterase, in order to remove exposed fatty-acid residues. Adherence will be assayed as described above.

1c. Inhibition of de novo synthesis of fatty-acids should reduce the fatty-acid content of emulsan and inhibit adherence phenomena.

Experimental - The exogenous addition of fatty-acyl-coenzyme A has been shown to inhibit de novo synthesis in bacteria, without affecting cell viability (33). The effect of various concentrations of hexadecanoyl-Coenzyme A on the adherence of RAG-1 to non-metabolizable hydrophobic surfaces will be tested during growth on soluble carbon and energy sources (see also section 3).

2. Following transfer of cells from conditions which do not favour adherence to conditions which do favour bacterial attachment, a lag should be observed. The addition of inhibitors of oxidative phosphorylation should inhibit adherence when added during the lag period but should not desorb cells which have already become attached.

Experimental - Inhibitors such as cyanide and azide will be added at various times following the transfer of cells and the degree of adherence will be measured.

3. Hydrophobic non-metabolizable solid surfaces may be substituted for the metabolizable hydrocarbon surface as substrata to which the bacteria adhere. Such surfaces include various polymers as well as hydrocarbons which the bacteria cannot degrade. Cells may thus be "tricked" into adhering to such surfaces as if they were metabolizable. Such a model system will enable us to study the effect of soluble carbon and energy sources on adherence phenomena.

Why model good: 1) separates metabolism from growth
2) enables counting
3) eliminates emulsion problems
4) enables working in high surface/vol ratio

Experimental - The experimental procedure will be that of Fletcher and Loeb (11), with the major exception that growth media will be employed, in addition to buffers.

4. Mutants deficient in their ability to adhere to hydrophobic surfaces can be isolated. Such mutants may not be able to grow in the presence of bulk hydrocarbon phase, as the sole carbon and energy source, but may be able to grow on finely emulsified hydrocarbon. It will be interesting to see whether:
   a) adherence-deficient mutants are also deficient in emulsan synthesis;
   b) mutants deficient in emulsan synthesis (certain classes of these mutants have already been isolated) are also deficient in their adherence ability;
   c) the non-adherence phenomena of the mutants can be reversed by the exogenous addition of emulsan.

Experimental - Mutants deficient in their ability to adhere to the hydrocarbon surface during growth will be isolated in the following manner: Mutagenized cells will be grown in a medium containing sufficient concentration of soluble carbon and energy substrate to permit growth yet not affect adherence ability? To this medium either toxic hydrocarbon or hydrocarbon containing a paraffin-soluble, water-insoluble bacterial toxin will be added. The assumption is that adherent cells will come into contact with very high concentrations of the toxin and be selectively killed, while non-adherent cells will survive.

Hydrocarbon hexane

Or toxin-resistant mutants would arise.

5. Clusters of bacteria observed on the hydrocarbon surface (see page 5) are the descendents of a solitary, adherent cell.

Experimental- One way to test this hypothesis is through the use of a mixed culture. RAG-1 and UP-2, for example, can be grown on the same medium on the same hydrocarbon source. Since these two strains can be distinguished visually, the clusters will be observed to be either homogeneous or heterogeneous.

how?

A more rigorous approach involves the use of a mixture of RAG-1 wild type and auxotroph (for amino-acid, for example), grown on hydrocarbon adsorbed to a membrane filter. After sufficient time is allowed for development of clusters, the filter paper is replicated onto selective plates which can distinguish the wild type from the auxotroph. In this manner the degree of homogeneity of the clusters can be ascertained.

It would be very difficult to determine heterogeneity on microcolonies after replication.

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Chem Abstract

89 120632- R2.

See pg 84
502 in solution

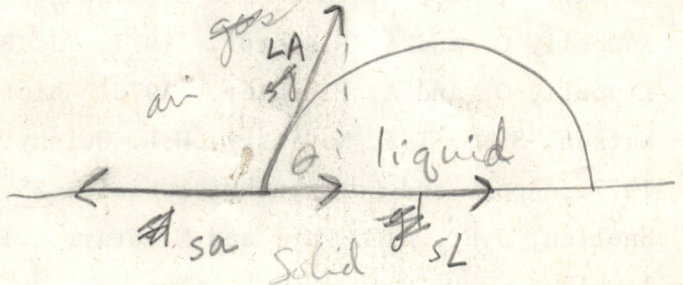
pg 89 silicone

~~Ilana's Method~~

30 cells/square

$30 \times 5 \times 10^5 =$

$150 \times 10^5 = \boxed{1.5 \times 10^7}$.



tensions act in the direction of the surfaces.

sg
sl
gl